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REMARKS

I. Status of the Application

Claims 10, 13, 14, 16-18, 20-23, 25, 26 and 28 are pending in the application. Applicants gratefully acknowledge the Examiner's withdrawal of her rejections of claims 10 and 13-19 under 35 U.S.C. § 112, first paragraph for written description; claims 10, 13-16, 18, 20, 22 and 23 under 35 U.S.C. § 102(b) as anticipated by Gazit et al; and claims 25, 26 and 28 under 35 § U.S.C. 102(b) as anticipated by Gleave et al. Claims 10, 13, 14, 16-18, 20-23, 25, 26 and 28 stand rejected under 35 U.S.C. § 112, first paragraph, as lacking enablement.

Applicants respectfully request entry and consideration of the foregoing remarks, which are intended to place this case in condition for allowance.

II. The Pending Claims Are Enabled

At page 3, paragraph 1 and page 5, paragraph 1 of the instant Office Action, the Examiner rejects claims 10, 13, 14, 16-18, 20-23, 25, 26 and 28 under 35 U.S.C. § 112, first paragraph, as lacking enablement. The Examiner is of the opinion that the claims contain subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains to make and/or use the invention. The Examiner's rejection is based on her view that due to a lack of direction/guidance, the absence of working examples, the complex nature of the invention and the breadth of the claims, undue experimentation would be required of the skilled artisan to make and/or use the claimed invention in its full scope. Applicants respectfully traverse these rejections.

35 U.S.C. § 112, first paragraph requires that the specification must enable a person skilled in the art to make and use the claimed invention. However, a specification need not, and

should not, disclose what is well known in the art. The invention that one skilled in the art must be enabled to make and use is that defined by the claims of the particular application. The issue of adequate enablement depends on whether one skilled in the art could practice the claimed invention without undue experimentation. Enablement is not precluded by the necessity of some experimentation such as routine screening, even if it is extensive routine screening. Also, the fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation (MPEP 2164.01) if the level of skill in the art is high or if all of the methods needed to practice the claimed invention are well known. *In re Wands*, 8 U.S.P.Q. 2d 1400, 1406 (Fed. Cir. 1988).

> The determination of what constitutes undue experimentation in a given case requires the application of a standard of reasonableness, having due regard for the nature of the invention and the state of the art. (Citations omitted). The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. *In re Wands*, 8 U.S.P.Q. 2d at 1404.

Applicants respectfully submit that the instant specification provides considerable direction and guidance to make and use the claimed invention. Applicants' claimed methods of producing active/growth factors require that one of skill in the art (1) apply undifferentiated cells on a substrate, (2) contact the cells with a culture medium, (3) induce the undifferentiated cells to differentiate and produce active/growth factors and (4) recover active factors from the culture medium. Each of these steps uses techniques that are well known by those of skill in the art in fields such as cell culture, cell biology, transplantation and the like. Further, Applicants provide several working examples wherein undifferentiated cells are added to a substrate in the presence

of culture medium, and the cells are induced to differentiate (e.g., Examples 1, 2 and 3) and wherein active factors are recovered (e.g., Example 4).

The Examiner asserts that undue experimentation would be required to induce undifferentiated mammalian cells to differentiate (paragraph bridging pages 4 and 5). Applicants respectfully disagree. Applicants submit that one of skill in the art would be able to use a variety of undifferentiated cells as methods of inducing many kinds of undifferentiated mammalian cells to differentiate were well known at the time of filing. For example, Biesecker et al. ((1993) *Proc. Natl. Acad. Sci, USA* 90:7044, set forth as Appendix A) teaches a technique for inducing embryonic stem cells to differentiate into embryonic lineages such as hematopoietic cells, cardiac monocytes and neurons (page 7044, left paragraph; page 7044, right paragraph, Methods section). Cumano et al. ((1993) *Proc. Natl. Acad. Sci, USA* 90:6429, set forth as Appendix B) teaches a procedure for inducing B-cell precursors to differentiate into immunoglobulin secreting cells (page 6429, abstract; page 6430, paragraph bridging left and right columns). Thus, one of skill in the art could practice the claimed invention using a variety of cells using methods known in the art at the time of filing.

The Examiner further asserts that undue experimentation would be required to induce stromal cells to differentiate, although the Examiner admits that the specification "teaches use of bone marrow cells in the claimed method" (page 6, full paragraph). Applicants respectfully submit that stromal cells ***are*** bone marrow cells. Applicants respectfully direct the Examiner's attention to page 2, lines 23-24, where Applicants teach bone marrow cells include hematopoietic cells such as stromal cells. Applicants submit that since they teach the use of bone marrow cells in the claimed method and since stromal cells are bone marrow cells, it logically follows that Applicants teach the use of stromal cells in the claimed method.

The Examiner further asserts that undue experimentation would be required to recover factors from the culture medium (paragraph bridging pages 4 and 5). Applicants respectfully disagree and submit that factors could be isolated using of variety of methods known in the art. For example, the culture media could be dialyzed to separate unidentified active factors from the media or the factors could be separated from the media by centrifugation. Alternatively, known factors could be separated by techniques such as affinity chromatography.

The Examiner also asserts that undue experimentation would be required to screen for activity of the factors (paragraph bridging pages 4 and 5). Applicants disagree. The pending claims are not directed to screening activity of the factors. Even so, Applicants respectfully submit that determining whether a factor is active could be easily achieved by assaying, for example, a specific cell morphology in the presence of the factor. Biesecker et al. teaches visual inspection of cells after induction to determine cell morphologies associated differentiation such as the formation of embroid bodies, cystic embroid bodies, cystic embroid bodies with primitive blood islands and cardiac tissue (Appendix A, page 7044, right column, Methods section). Cumano et al. teaches visually noting a change in B-cell precursor structure to acquire a particular morphology that is unique to differentiated cells (Appendix B, page 6430, paragraph bridging left and right columns). Immunological assays such as antibody binding could also be used to determine activity of the factor. Cumano et al. (Appendix B), for example, teaches the use of ELISA assays to determine differentiation (page 6430, left column, ELISA Assays section).

For at least the reasons set forth above, Applicants' specification, coupled with the level of skill knowledge in the art of cell culture enables one skilled in the art to make and/or use the claimed invention. Accordingly, the Examiner is respectfully requested to reconsider and

withdraw the rejection of claims 10, 13, 14, 16-18, 20-23, 25, 26 and 28 under 35 U.S.C. § 112, first paragraph.

III. Conclusion

Having responded to all outstanding issues, reconsideration and allowance of all the pending claims is respectfully requested. If a telephone conversation with Applicants' attorney would expedite prosecution of the above-identified application, the Examiner is urged to call the undersigned at (617) 720-9600.

Respectfully submitted,

Dated: April 14, 2004

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Developmental Biology

Identification of four murine cDNAs encoding putative protein kinases from primitive embryonic stem cells differentiated *in vitro*

(receptor/hematopoiesis/embryo/mouse)

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ABSTRACT **Protein kinases transduce signals from extracellular ligands in the hematopoietic and other systems through direct phosphorylation of tyrosine, serine, or threonine residues. Little is known about the ligands and receptors that are important in the earliest stages of development—i.e., stem cell self-renewal and lineage commitment. We have made use of the lineage differentiation potential of the murine embryonic stem cell system to clone partial cDNAs encoding four putative protein kinases. Three of the four genes contain the highly conserved residues Asp-Phe-Gly in domain VII of the protein kinase family. These genes are candidates for receptors or downstream effectors of cytokines that regulate self-renewal and lineage commitment in embryogenesis.**

Much effort has been directed toward isolation and study of primitive stem cells in several tissues, both to understand the biology of these cells and to use them clinically in transplantation and gene therapy (1). Despite real progress on understanding the regulation of the differentiation of mature lineage-specific cells (1, 2), little is known about the process of primitive stem cell self-renewal and function. For example, in the hematopoietic system, while much is known about cytokine regulators of precursor and progenitor cell differentiation, the molecules that regulate hematopoietic stem cell (HSC) self-renewal and lineage commitment are unknown (3).

The embryonic stem cell (ESC) *in vitro* differentiation system provides an opportunity for the study of early developmental events in mammals (4). This system models the transition from pluripotent embryonic cells (blastocyst inner cell mass) to embryonic lineages, including hematopoietic cells (yolk sac blood islands), cardiac myocytes, neurons, and others. In addition, it has been demonstrated that a soluble factor (or factors) present in human umbilical cord serum (HUCS) can alter this developmental cascade, suggesting that ligand–receptor interactions are involved in this process.

The information for the coordinated differentiation, cell replication, migration, and cell death required for normal differentiation is transmitted within and between cells by a number of mechanisms. Ligand–receptor pairs are a major component of intercellular communication in the developing and mature animal (5). Several of these ligands mediate their function through receptors that belong to the protein kinase family (6). Protein kinases are enzymes that catalyze the transfer of phosphate from ATP to tyrosine, serine, or threonine residues on the receptor or proteins that are downstream in a signaling cascade. The receptor protein kinases catalyze this reaction when a ligand is bound to the extracellular domain, triggering a cascade of events leading to an alteration in cell function such as proliferation or migration. The protein kinase family numbers greater than 100 members, including oncogenes, contractile proteins, cell cycle proteins, viral kinases, developmental proteins, and cell surface receptors (6). We hypothesized that additional related protein kinases may be involved in more primitive lineage-specific stem cell regulation.

On the basis of this hypothesis an experimental strategy was designed to isolate novel protein kinase sequences from embryonic tissues *in vitro* by using the DNA sequence homology of the protein kinase family. Specifically, we have isolated such sequences from murine ESCs in culture at the time that these cultures are known to be producing lineage-specific stem cells. These sequences are candidate protein kinase cell surface receptors or intracellular signaling molecules whose regulation activates proliferation and differentiation of stem cells in the early embryo.¶ These partial cDNA sequences will be directly applicable for cloning full-length cDNAs that can be analyzed to determine their functions in intra- and intercellular signaling, the upstream and downstream effector molecules, the ligands for those found to be receptors, and their role in mammalian development and adult homeostasis.

METHODS

Cell Culture and Differentiation. The embryonic stem cell line ES-D3 (4) was used in all experiments. The cells were cultured in Dulbecco's modified Eagle's medium (Irvine), 15% heat-inactivated fetal calf serum, leukemia inhibitory factor (7) at 1000 units/ml (GIBCO/BRL), and 150 μM monothioglycerol (8) in flasks that were treated with 0.1% 300-bloom porcine gelatin (Sigma). To induce differentiation, colonies were trypsinized and transferred to bacterial culture dishes in media without leukemia inhibitory factor and with 20% fetal calf serum, and 450 μM monothioglycerol. After 10 days the medium was changed to Iscove's modified Eagle's medium/20% heat-inactivated HUCS/450 μM monothioglycerol. The cultures were inspected every second day and the simple embryoid bodies, cystic embryoid bodies (CEBs), and CEBs with primitive blood islands (PBIs) or cardiac tissue were tabulated. Individual CEB-containing PBIs were selected with microscopic visualization. These PBI-containing CEBs were lysed with 4.0 M guanidinium isothiocyanate, 0.1 M Tris·HCl, pH 7.5, and 1% 2-mercaptoethanol and frozen. The lysates

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Abbreviations: ESC, embryonic stem cell; CEB, cystic embryoid body; PBI, primitive blood island; HUCS, human umbilical cord serum.

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¶The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L10913 for ETK-1, L10914 for ETK-2, L10915 for ETK-3, and L10916 for ETK-4).

were pooled and total RNA was isolated by centrifugation over cesium chloride (9, 10).

HUCS. Umbilical cord blood was collected under a protocol approved by the University of Michigan Institutional Review Board in serum separator tubes from the maternal end of the cut cord. The tubes were centrifuged at 3000 × *g* and the serum was pooled and recentrifuged to remove any erythrocytes from the serum. Specimens with visible hemolysis were discarded.

Reverse Transcription and PCR. One microgram of total RNA from hematopoietic CEBs or ESCs was reverse transcribed by using Molony murine leukemia virus reverse transcriptase (GIBCO/BRL) and random hexanucleotides according to supplier's instructions. One-fifth of the cDNA product of the above reaction was amplified with *Taq* DNA polymerase, using degenerate primers that contain restriction enzyme sites on the 5′ ends (11). The primers hybridize to conserved protein kinase domains VI and IX and include between them two additional conserved domains (VII and VIII). In addition, the primers flank less conserved domains that allow for the discrimination of novel genes. The amplification parameters were: 95°C for 1.5 min, 37°C for 2 min, and 63°C for 3 min for 50 cycles, followed by 10 min at 63°C. All reagents except cDNA and buffer were replenished after 25 cycles.

Cloning and Sequencing. PCR products were separated by gel electrophoresis and ligated into pUC18. Sequencing of double-stranded plasmids was performed with Sequenase version 2.0 (United States Biochemical) following the supplier's instructions (12). Sequence analysis was performed with the Genetics Computer Group program (13).

Northern Blot Analysis. Total RNA was prepared by detergent lysis from C57BL/6 or C57BL/6 × SJL F_1 mice (The Jackson Laboratory) under a protocol approved by the University [of Michigan] Committee for the Use and Care of Animals. Poly(A)$^+$ RNA was isolated by affinity chromatography with oligo(dT)-cellulose (Invitrogen). The RNA was separated by electrophoresis in 1.0% agarose with 17% formaldehyde and transferred onto nylon membranes. The Northern blot of adult tissues was acquired from Clontech. The ETK- and human β-actin-containing plasmids were labeled with [α-^{32}P]dCTP by random hexanucleotide priming (14) and hybridized to the membranes at 42°C overnight. Blots were washed two times at room temperature in 2× SSC/0.1% SDS and then two times at 50°C in 0.1× SSC/0.1% SDS (1× SSC = 150 mM NaCl/15 mM sodium citrate, pH 7.0). Blots were exposed to Kodak X-AR film in cassettes with Cronex intensifying screens.

RESULTS

ESCs were cultured under conditions to induce tissue-specific differentiation (Fig. 1). The cells were cultured for 10 days in 20% fetal calf serum and then transferred to HUCS. Between days 13 and 17, 35% of the resulting CEBs contained islands of hematopoiesis, and 30% contained actively beating cardiac muscle, in agreement with previous data (4) (Fig. 1). PCR amplification of reverse transcribed RNA from these hematopoietic embryoid bodies yielded the expected amplification product of 210 bp as described by Wilks (11). This PCR product was cloned in pUC18, which was used to transform *Escherichia coli*, and DNA from 93 colonies was analyzed. Seventy of these clones had an appropriate size insert and were sequenced. Ten previously described tyrosine kinase genes were represented in these clones, including FD17, FD22, murine platelet-derived growth factor receptor, murine fibroblast growth factor receptor, murine *abl* oncogene, murine hematopoietic cell kinase, fetal liver kinase 1, murine *fyn* oncogene, murine *Met* oncogene, and murine insulin receptor (Table 1). No previously described nonkinase cDNAs were identified in these experiments. In addition to these previously described kinases, four apparently novel cDNAs (designated ETK-1 through ETK-4) encoding proteins with significant amino acid similarity to domains VII and VIII of protein kinases were also cloned three to six times each from the differentiating ESCs (Fig. 2).

The similarity of the four partial ETK cDNA clones to known DNA sequences is listed in Table 2. ETK-1 is most similar to an unpublished sequence in GenBank designated hump78 (accession no. X57019) but no further information is available. The high degree of similarity of ETK-1 to this human sequence suggests that ETK-1 is the murine equivalent of hump78. ETK-2 is most similar to a putative tyrosine kinase receptor designated UFO (15) or JTK11 (18). The degree of similarity between ETK-2 and UFO/JTK11 is much lower than that of ETK-1 and hump78, suggesting that

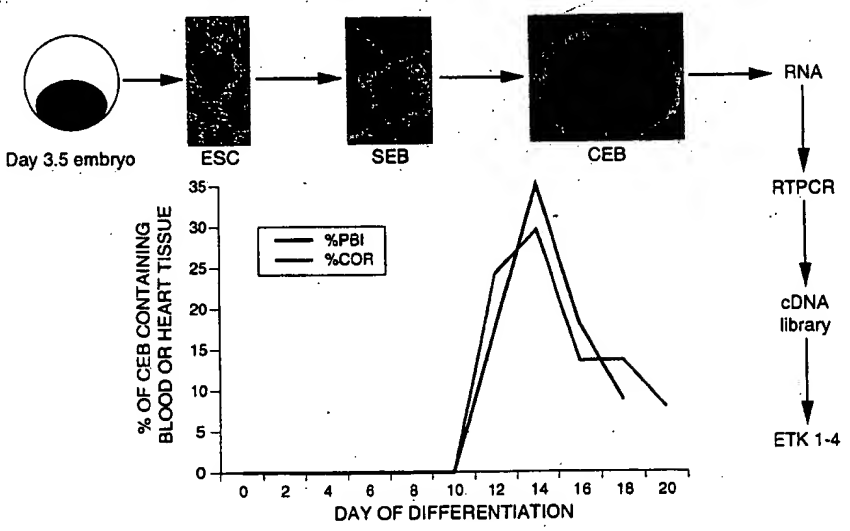

FIG. 1. Experimental design. ESCs were induced to differentiate in HUCS-containing medium. SEB, simple embryoid body. RNA was purified from the differentiated ESCs and amplified with protein kinase primers. RTPCR, reverse transcription PCR. Time course of differentiation of ESCs in days. The ordinate shows the percentage of embryoid bodies that contained the indicated structure [PBIs or cardiac tissue (COR)].

Table 1. Known protein kinases cloned from differentiating ESCs

Gene	Frequency*
FD17	37
Platelet-derived growth factor receptor	5
Hematopoietic cell kinase	3
Fetal liver kinase 1	2
Fibroblast growth factor receptor	2
abl	2
FD22	2
fyn	1
Met	1
Insulin receptor	1

*Frequency indicates the number of clones of the indicated sequence that were found among 56 known tyrosine kinases cloned from differentiated ESCs.

Table 2. Homology of ETK genes

Gene	Closest homologue*	DNA similarity† % identity	DNA similarity† Length, bp	Protein similarity† % similarity	Protein similarity† Length, aa
ETK-1	hump78	93	178	96	60
ETK-2	Human tyrosine kinase	61	141	40	88
ETK-3	Human *MET* oncogene	68	152	42	53
ETK-4	*Drosophila ninaC*	71	146	45	53

*The four ETK genes are listed with the closest homologue identified by the FASTA algorithm in the GenBank data base (13). The references for the homologues are hump78 (GenBank accession no. X57019), human tyrosine kinase (15), human *MET* (16), and *Drosophila melanogaster ninaC* (17).

†The homology columns indicate the length of the segment that was matched in the data base and the frequency of an exact match (DNA) or similarity (protein).

ETK-2 is related to this human gene but is not the murine equivalent. ETK-3 is most similar to the human *MET* oncogene (16). Interestingly, ETK-3 does not share the Asp-Phe-Gly triplet motif that is present in nearly all protein kinases and is thought to be involved in ATP binding. It will be interesting to learn if this protein is capable of transferring phosphate to tyrosine, serine, or threonine residues. ETK-4 is most similar to the *Drosophila ninaC* protein kinase (17).

These partial cDNA sequences have been designated as putative protein kinases because of the sequence similarities to the protein kinase family, although confirmation of kinase activity awaits further experimental data. ETK-1 and -4 are most similar to the serine/threonine class of protein kinases because of the presence of a lysine in position 1 for both sequences and a tyrosine in position 39 of ETK-1 and a threonine in position 37 of ETK-4. ETK-2 and -3 are most similar to the tyrosine kinase family because of the presence of an alanine in position 1 and the proline-valine doublet at positions 39 and 40.

Expression studies were performed by Northern blot analysis of poly(A)$^+$-selected RNA. The tissues used for this screening included adult and fetal (day 15) liver, thymus, heart, spleen, muscle, kidney, brain, testes, and lung (Figs. 3 and 4). ETK-1 is widely expressed in adult tissues and fetal liver, with the highest expression in kidney (Figs. 3*A* and 4*A*). The Northern blot data suggest that there are alternative transcripts of 3.5 and 3.0 kb in many tissues or that the probe cross-hybridizes with a related transcript. The ETK-2 mRNA is approximately 3.9 kb in length, with high expression in brain, moderate in testes, and low in lung tissue and undifferentiated ESCs (Figs. 3*B* and 4*B*). The ETK-3 mRNA is expressed in all of the tissues tested, similar to ETK-1 (except for thymus), and the probe hybridizes to transcripts of 2.7 and 3.0 kb (Figs. 3*C* and 4*C*). It is interesting that the larger of the ETK-3 transcripts has a size and expression pattern that is similar to that of the smaller ETK-1 transcript, suggesting either that the probes cross-hybridize with both messages or that one or both of these genes produce alternative transcripts. The fact that ETK-3 does not hybridize to thymus mRNA suggests that alternative splicing is more likely. The ETK-4 message is expressed in brain, heart, lung, kidney, ESCs, and fetal liver. The ETK-4 probe hybridizes to transcripts of 8.1, 6.3, and 4.8 kb. The 8.1-kb transcript is prominent in the adult tissues, but all three transcripts are of similar intensity in the ESCs, fetal liver, and testes (Figs. 3*D* and 4*D*). These data suggest that additional homologous cross-hybridizing transcripts are present in the embryonic tissues, or the ETK-4 transcript undergoes differential splicing in testes and embryonic tissues. The length of the ETK-4 transcript suggests a significantly different protein structure

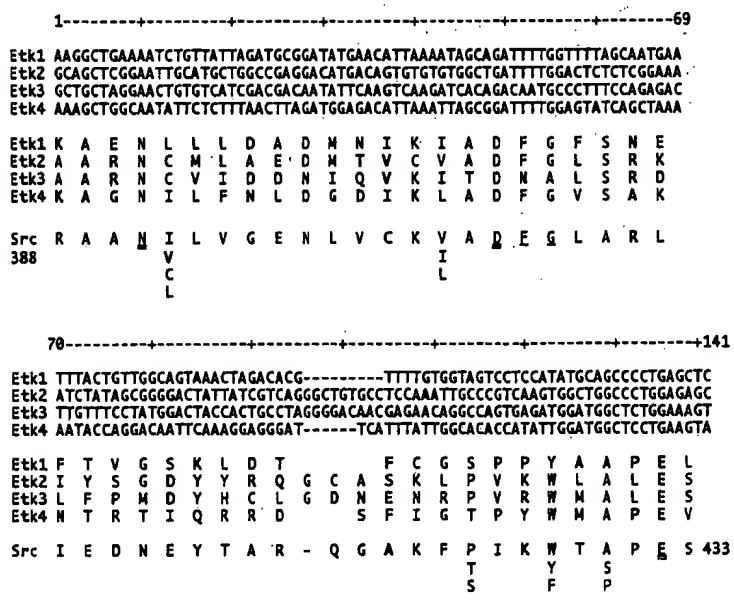

FIG. 2. DNA and protein sequence comparisons of ETK-1, -2, -3, and -4 and Src. Two underlines in a position indicate a highly conserved residue. One underline indicates a structurally similar residue conservation. Additional residues below the Src sequence indicate other common amino acids present at that position as described by Hanks *et al.* (6). Numbering convention is for the chicken Src kinase, although the human Src sequence is shown.

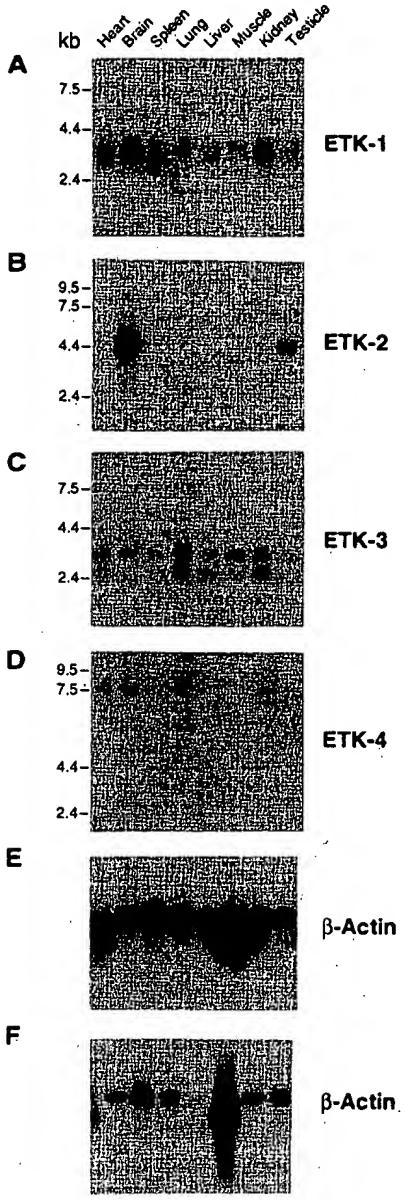

FIG. 3. Northern blot analysis of ETK expression in adult mice. Each lane of the Northern blot contains 2 μg of poly(A)$^+$-selected RNA. (*A*) ETK-1 probe, exposed 5 days. (*B*) ETK-2 probe, exposed 3 days. (*C*) ETK-3 probe, exposed 7 days. (*D*) ETK-4 probe, exposed 7 days. (*E*) Human β-actin probe, exposed 5 hr. (*F*) Human β-actin probe exposed 12 hr. ETK-1, -2, and -3 were analyzed on a single Northern blot filter and ETK-4 was analyzed on a second filter. The β-actin equalization signals are shown in *E* and *F* for the first and second filters, respectively.

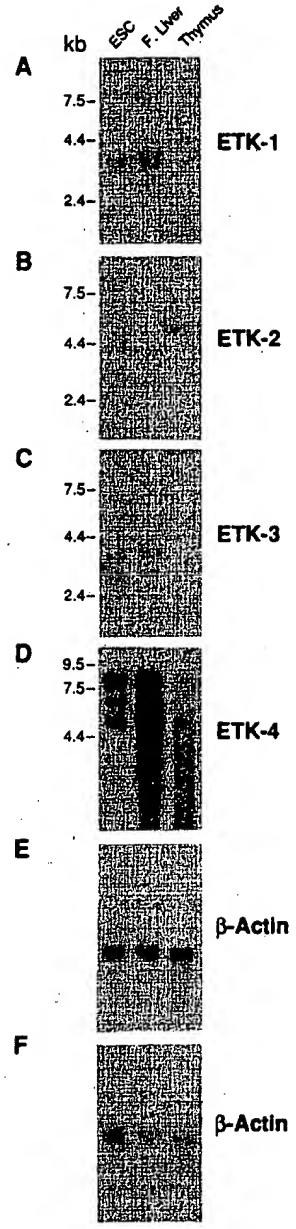

FIG. 4. Northern blot analysis of ETK expression in undifferentiated ESCs, fetal liver (day 15), and adult thymus. Each lane contains 2 μg of poly(A)$^+$-selected RNA. (*A*) ETK-1 probe, exposed 3 days. (*B*) ETK-2 probe, exposed 3 days. (*C*) ETK-3 probe, exposed 3 days. (*D*) ETK-4 probe, exposed 3 days. (*E* and *F*) Human β-actin probe, exposed 16 hr. The β-actin control filter for ETK-1 and ETK-3 is shown in *E* and that for ETK-2 and ETK-4 is shown in *F*.

than ETK-1, -2, and -3, perhaps coding for a larger transmembrane or extracellular domain.

DISCUSSION

Protein kinases play key roles in cell-to-cell signaling and intracellular homeostasis, regulating many important functions of homeostasis (6). Transmembrane receptor kinases fulfill this function in the developing organism for several ligand/receptor pairs, including platelet-derived growth factor receptor, sevenless, epidermal growth factor receptor, c-kit, and others (19–22). To characterize the regulation of primitive lineage-specific stem cell proliferation we chose to attempt to isolate additional growth factor receptors. The experimental design used the *in vitro* ESC differentiation system because we are interested in genes that are active in stem cell turnover and lineage commitment, and such events are less common in the mature organism. The *in vitro* differentiation potential of the ESCs was used in these experiments as a model of the differentiation process [embryonic day 4 to day 10 in the mouse (4)] to clone partial

cDNAs that encode putative developmental growth factor receptors. This strategy is based on the hypothesis that tissue-specific stem cells must differentiate from pluripotent precursors early in development, and the signals that trigger this differentiation are the same as those that are responsible for stem cell turnover in the adult.

Degenerate primers were used to amplify partial sequences of four previously undescribed transcripts that have sequence similarity to the protein kinase gene family (6). These sequences have been designated as putative kinases because they are similar in deduced amino acid sequence to the family of protein kinases. Confirmation of the biochemical activity of these proteins will require demonstration of auto- or heterophosphorylation of tyrosine and serine/threonine residues. Three of the four ETKs contain all the highly conserved amino acid residues present in protein kinase domains VI–IX. Interestingly, ETK-3 has a variation in two of the highly conserved amino acid residues in subdomain VII that is thought to be responsible for ATP binding (6) (Fig. 2). This variant sequence is also found in the *trk* tyrosine kinase gene from *Drosophila* (23). Not all protein kinases are transmembrane receptors, and the receptor domains were not cloned in these experiments, leaving open the question of whether these putative protein kinases are in fact receptors.

The expression patterns of the ETK genes suggest different roles for these putative kinases. The broad expression patterns of ETK-1 and ETK-3 are consistent with a protein that functions in the turnover and maintenance of committed progenitor cells. These genes may function in a cell that is common to many tissues (connective tissue, vascular endothelium, etc.), or the gene may function in many cell types, both possibilities yielding a broad tissue expression pattern. This broad expression pattern of ETK-1 and ETK-3 does not preclude a role for this gene in tissue-specific developmental processes, as a gene can have very different roles in adult and embryonic tissues. In contrast, ETK-2 has a highly restricted expression pattern, being present only in brain, lung, ESCs, and testes, of the tissues so far examined. The expression of a tyrosine kinase in brain and testes would be consistent with a receptor that functions in a neuroendocrine signaling pathway in the adult, or the receptor may be expressed in a cell type that is common to these tissues. The predominant ETK-4 transcript in adult tissues is larger than ETK-1 to -3 transcripts but is similar in size to the transcript of the fetal liver kinase gene (24), another member of the protein kinase family. The ETK-4 Northern blot data suggest that the longer ETK-4 transcript predominates in adult tissues, but the relative level of the smaller transcripts is higher in primitive and germ cells. ETK-4 also has restricted tissue expression, with moderate expression in heart, brain, ESCs, fetal liver, and kidney, and low levels in spleen, liver, and testes. This expression pattern suggests that the ETK-4 gene plays a role in a variety of tissues in the adult or, as suggested above for ETK-1 and -3, is present in a cell type that is common to these tissues. The hybridization pattern on the ETK-4 Northern blots suggests that ETK-4 or a closely related tyrosine kinase plays a role in the function of these tissues, possibly acting as a signaling effector for generation or turnover of primitive stem and germ cells in these tissues.

These four partial cDNA sequences have similarity to the family of protein kinases and are expressed in ESCs, fetal liver, and in a variety of adult tissues. The cloning of these partial cDNA sequences allows the characterization of the complete sequences of these molecules to determine if they have structural characteristics of receptors, intracellular signaling molecules, or other classes of effector molecules. All of the ETK genes are also candidate oncogenes. Dysregulation or structural rearrangement of these genes may lead to perturbations of cell differentiation or growth that could cause or participate in neoplastic transformation. It will be important to determine the effects of up- and down-regulation of these genes on cultured cells and on differentiation *in vitro* and *in vivo*. These data will elucidate mechanisms of differentiation that are relevant to basic questions of mammalian development and clinical problems such as transplantation, gene therapy, and oncogenesis.

Note. Subsequent to the submission of this manuscript we have updated the homology data for the ETK genes based upon recent GenBank searches or references that contain sequences not entered in the data bases. ETK-1 is similar to murine *Mpk-10* (99.4% nucleotide identity; GenBank accession no. X57244). ETK-2 is identical to rat tyro-3 (44 amino acid residues) (25); ETK-3 is identical to *RYK* (M98547) and *vik* (L02210); and ETK-4 is similar to human SK2 (97.8% amino acid identity) (26).

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Immunology

Differentiation and characterization of B-cell precursors detected in the yolk sac and embryo body of embryos beginning at the 10- to 12-somite stage

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ABSTRACT **The embryonic sites in which progenitors of the hematopoietic lineages first emerge are ideal regions to characterize both the cells and environment needed to initiate blood cell development. For a number of years both the murine yolk sac and embryo have been recognized to contain progenitors of B lymphocytes. However, clonal, quantitative *in vitro* assays, which allow precise observation of precursors and their progeny, have been lacking. Moreover, the site of origin of the initial events remains controversial. In this report we document the presence of B-cell progenitors in yolk sac and embryonic tissue obtained from mouse fetuses beginning at the 10-somite stage, day 8.5. We determine the frequency, cell-surface phenotype, and growth properties of these progenitors. We show that these cells can differentiate into immunoglobulin-secreting cells and that the progeny derived from single progenitors are diverse with respect to immunoglobulin heavy-chain allotype expression, diversity–joining region use, and heavy-chain variable-region utilization.**

The dominant site of murine embryonic B lymphopoiesis is the fetal liver. On day 10–11 of gestation, the fetal liver is seeded by progenitors of unknown origin. The characterization of these progenitors is critical for understanding the process of lineage commitment and identification of the factors that regulate this process. A number of potential origins have been examined during embryonic development, including yolk sac, omentum, placenta, embryonic blood, liver, and undefined portions of the embryo (1–6).

In birds it has been demonstrated that the embryo and the yolk sac are independently seeded by hematopoietic precursors and that the precursors found in the embryo are responsible for adult hematopoiesis (7). Recently, it has been reported that the first immunoglobulin gene rearrangements are found in the yolk sac (8). In mouse, *in vivo* reconstitution experiments showed hematopoietic reconstitution potential in the yolk sac and in the embryo body at day 8 and 9 of gestation (1). Cells capable of undergoing subsequent immunoglobulin gene rearrangement *in vitro* arise in embryo bodies at day 9 of gestation (9).

Mature hematopoietic cells have not been observed in yolk sac blood islands, although myeloid colonies have been cloned from yolk sac precursors with *in vitro* assay conditions (10). In contrast, lymphopoiesis has not been seen before the fetal liver stage of differentiation, and surprisingly, yolk sac-derived progenitors failed to give rise to mature B cells *in vitro*. This result has led to the suggestion that the developmental potential of yolk sac precursors might be limited.

In this report we describe the generation of mature B lymphocytes from hematopoietic precursors in the yolk sac and embryo body at day 8–10 of gestation. We used a recently described clonal assay that allows the development of uncommitted hematopoietic precursors to generate B lymphocytes (11–13). We detect as many as 100 B-cell progenitors that can differentiate to immunoglobulin-secreting cells in one embryo. Moreover, we find that precursors are present both in the yolk sac and in the body of the embryo. These cells express the surface marker AA4.1, although they cannot be isolated on the basis of Ly6A (Sca1) antigen expression. These progenitors proliferate extensively *in vitro*, undergo heavy- and light-chain immunoglobulin gene rearrangements, and express diverse variable-region genes.

The results demonstrate that B-cell precursors can be detected as early as day 8.5 of gestation in both yolk sac and the embryo body, showing that lymphopoietic precursors can be found outside the liver. The identification of a surface marker in such an early stage of development will allow us to determine the origin and follow the fate of those precursor cells. In addition, the enrichment of the precursor population based on the surface markers will allow us to ascertain the differentiation potential of those cells at the clonal level.

MATERIALS AND METHODS

Cell Preparations. C57BL/6 mice and BALB/c mice were purchased from The Jackson Laboratory. Timed pregnancies were obtained by housing two to three female mice with one male for 18 hr. Detection of vaginal plugs confirmed mating (day 0). Mice were sacrificed by cervical dislocation on the morning of day 8–10. Yolk sac and embryo body cell suspensions were obtained by dissecting the tissues using a low-power stereoscope and dispersing them by passage through a 26-gauge needle. Viable cells were determined by trypan blue exclusion.

Culture Conditions. The *in vitro* culture conditions were the same as described in detail (12, 13). Briefly, S17 stromal cells (14) were plated in either 96-well plates (500–1000 cells per well) or 24-well plates [2500–5000 cells per well (Costar)] in complete medium (see below). S17 cells were cultured at 37°C, 5% CO_2 overnight before irradiation (*in situ*) with 2000 Gy using a cesium source. Single-cell suspensions of yolk sac or embryo midbody cells were seeded in the plates with interleukin 7 (IL-7) at 50–100 units per ml, obtained from the supernatant of stably transfected cells (from Fritz Melchers, Basel). Cells were seeded at limit dilution by using 48–96 wells for each cell concentration. Every 5 days half of the medium was replaced by fresh medium containing IL-7. The mitogen stimulation assay was done by plating 5000 cells on irradiated S17 cells (2000 cells per well) in 96-well plates. Lipopolysaccharide (LPS: *Salmonella typhosa* W0901;

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Abbreviations: IL-7, interleukin 7; LPS, lipopolysaccharide; V_H, heavy-chain variable region.

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Difco) was added at 25 μg/ml. Freshly made OPTI-MEM (GIBCO) supplemented with 10% fetal calf serum (GIBCO), 2×10^{-5} M 2-mercaptoethanol, $NaHCO_3$ at 2.4 g/liter, streptomycin at 5 mg/liter, and penicillin at 5 units per ml was used for all experiments.

The double-layer agar assay for RNA colony analysis has been described in detail (15, 16). Briefly, a 1-ml solution containing OPTI-MEM supplemented as described above and also containing 0.3% melted agar (Difco) was allowed to gel and served as the bottom agar layer. A top layer consisting of the same ingredients and also containing cells was poured subsequently. After 5 days of culture (5% CO_2 at 37°C) the top layer was removed and transferred to a nylon membrane placed over a vacuum chamber. After applying vacuum, ≈20 ml of 2× standard saline citrate (SSC; 20× SSC is 3 M sodium chloride/0.3 M sodium citrate) containing 0.2% SDS (BRL Ultrapure) was pipetted over the agar disk. Subsequently, ≈2 ml of hot formamide (80°C) was used to dissolve the agar, after which the filters were allowed to dry thoroughly by using a heat lamp.

Hybridization and Probes. Filters were prehybridized for 6–12 hr at 42°C in 50% (vol/vol) formamide, 5× SSPE (20× SSPE is 3.6 M NaCl/200 mM NaH_2PO_4/20 mM EDTA, pH 7.4), 4× Denhardt's [50× Denhardt's is 1% Ficoll/1% poly(vinylpyrrolidone)/1% bovine serum albumin (Pentax fraction 5)]/1% SDS/polyadenyloic acid/salmon sperm DNA that had been previously denatured each at 100 μg/ml. Hybridization was done for 24 hr in the same solution, which also contained labeled probes (1×10^6 cpm/ml). Filters were washed twice with 2× SSPE/0.2% SDS for 30 min each at 42°C, twice with the same solution at 60°C, and once with 1× SSPE/0.2% SDS for 30 min at 60°C. The filters were autoradiographed with two intensifying screens at −70°C for 2–14 days. The probes used for detecting immunoglobulin μ heavy-chain and the variable-region families (J558, Q52, and 7183) have been described in detail (16).

Panning Procedure. Enrichment for cells by panning was done as described (11). Optilux 100-mm plastic Petri dishes (Falcon no. 1001) were coated with an affinity-purified mouse anti-rat IgG (50 μg per plate; Jackson ImmunoResearch) and subsequently incubated with saturating amounts of supernatant from rat hybridomas producing either anti-Ly6 (Sca-1) (17) or anti-AA4.1 (18) antibodies. Nonadherent cells were recovered by two gentle washes with ice-cold Earle's balanced salt solution (GIBCO)/5% fetal calf serum. Adherent cells were recovered by using a rubber policeman after an additional four washes.

ELISA Assays. Immunoglobulin production was monitored based on ELISA assays. NuncImmuno plate MaxiSorp (GIBCO) were coated with affinity-purified anti-mouse μ-chain antibody or allotype-specific hybridomas AF6 (anti-mouse Igh6b) or RS3.1 (anti-mouse Igh6a), as described (12). Plates were blocked with phosphate-buffered saline (PBS)/2% bovine serum albumin, for 1 hr at 37°C. Serial dilutions of the supernatants in PBS/2% bovine serum albumin were applied and incubated for 1 hr at 37°C. Plates were then incubated with appropriate dilutions of antimouse μ chain coupled to horseradish peroxidase (Sigma). Plates were then incubated with the substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), and the absorbency was read at 405/630 nm.

RESULTS

Frequency and Surface-Marker Expression in B-Cell Precursors Isolated from Yolk Sac and Embryos. Cells were obtained from the yolk sac and embryo body at day-9 and -10 gestation and tested in a limiting-dilution assay for B-cell progenitors, as described (12, 13). Cells were seeded in 96-well plates at concentrations of 3000, 1000, and 300 cells per well in the presence of irradiated S17 stromal cells and IL-7. Growth of small, nonadherent lymphocytes was scored after 10–14 days. The morphology of such clones is unique, and we have previously demonstrated that such cells express the pre-B surface antigen B220 and respond to LPS stimulation by immunoglobulin secretion (12). This result was confirmed again for aliquots of cells obtained in the experiments reported here.

The results of four independent experiments in which the frequency of B-cell progenitors found in the yolk sac and embryo was determined are shown in Table 1. Although both the yolk sac and embryo body contained progenitors, the numbers of these varied widely in individual experiments. For example, in experiments 2 and 3 the numbers of precursors detected in the yolk sac were substantially lower than in the embryo body, whereas in experiments 1 and 4, the number of precursors detected in the yolk sac was higher than in the embryo body. We detected between 45 and 100 precursors from each embryo at this time of gestation. By contrast, we found 385 and 1300 precursors, respectively, in the fetal liver at day 11 or 12 of gestation.

Care was taken to avoid contamination of cells from the two sources during the dissection of yolk sac and embryonic tissue. However, to ensure that cells are not obtained by nonspecific mixing we isolated the heads of the embryos from the same dissection plates. As shown in Table 1, B-cell precursors were absent from this cell preparation, although ≈30 precursors could be detected in the midportion of those embryos.

We next determined whether the cell-surface phenotype of yolk sac and embryo-derived B-cell progenitors was similar to that established for fetal liver-derived B-cell progenitors. We used both the AA4.1 and Ly6A/Sca-1 antibodies for this analysis. As shown in Table 1, $AA4.1^+$ cells (1.6–2.8% cells

Table 1. Number of B-cell precursors recovered from yolk sac and embryo body at days 9 and 10 of gestation

Exp.	Day of gestation	Tissue analyzed	B-cell precursors per embryo, no. (frequency)
1	10.0	Yolk sac	62 (1:700)
		Yolk sac $AA4.1^+$	50 (1:140)
		Yolk sac MaRIgG	<1 (<1:4,000)
		Yolk sac $Ly6A^+$	<1 (<1:18,000)
		Embryo body	30 (1:5,600)
		Embryo body $AA4.1^+$	30 (1:140)
		Embryo body MaRIgG	<1 (<1:2,000)
		Embryo body $Ly6A^+$	<1 (<1:24,000)
2	9.0	Yolk sac	12 (1:833)
		Embryo body	55 (1:545)
3	9.0	Yolk sac	2 (1:2,000)
		Embryo body	45 (1:666)
4	9.0	Yolk sac	64 (1:1,050)
		Yolk sac $AA4.1^+$	63 (1:130)
		Embryo body	33 (1:2,000)
		Embryo body $AA4.1^+$	26 (1:30)
		Embryo heads	<0.5 (<1:30,000)

Cells recovered from the different tissues were plated in 96-well plates with S17 cells irradiated with 2000 Gy and IL-7 (100 units/ml) at concentrations of 300, 1000, and 3000 cells per well. Cells enriched by panning for expression of surface markers were plated at concentrations of 6, 20, and 60 cells per well. Nonspecific plastic adherence was tested by analyzing cells adherent to a plate coated with mouse anti-rat IgG (MaRIgG). All clones identified as containing B-cell precursors by microscopic inspection were stimulated by LPS, and their ability to secrete immunoglobulin was tested by an ELISA assay. The total numbers recovered from yolk sac and embryo bodies were respectively 4.4×10^5 and 1.7×10^5 for Exp. 1, 10^4 and 3×10^4 for Exp. 2, 4×10^4 and 3×10^4 for Exp. 3, and 6.6×10^4 and 6.8×10^4 for Exp. 4. In this same experiment the number of cells recovered from each head was 1.6×10^4 cells.

recovered) contain virtually all precursors detected under these conditions, irrespective of whether they are isolated from yolk sac or embryo body. By contrast, no B-cell precursors were detected in the Ly6A$^+$ population (1.8–6% cells recovered). In an additional specificity control, we failed to find B-cell precursors among cells recovered from plates coated with mouse anti-rat immunoglobulin (<0.4% cells recovered).

The results described above indicate that B-cell precursors that differentiate *in vitro* to immunoglobulin-secreting cells can be detected as early as day 9.0 of gestation, and they can be found both in the yolk sac and in the embryo body. They express the surface-antigen AA4.1.

To determine whether or not the progenitor cells were present at earlier stages of development we examined embryos and yolk sacs on day 8.5 of gestation. At this stage of development the numbers of B-cell precursors detected were variable. In three independent experiments we found the following: Exp. 1 stage 10–12 somites, three B-cell progenitors per embryo body, and fewer than one B-cell progenitor per eight yolk sacs; Exp. 2 stage 10–12 somites, one B-cell progenitor per four embryo bodies, and one B-cell progenitor per two yolk sacs; and Exp. 3 stage 15 somites, one B-cell progenitor per two embryo bodies, and three B-cell progenitors per yolk sac. Five additional experiments were done by using day-8 embryos (<10 somites). We routinely failed to detect progenitors from yolk sacs or embryo bodies obtained at this stage of development. These data suggest that we are at the limit of detection and may well be observing the initial emergence of progenitors able to differentiate in our assay system. Moreover, this event occurs in both the yolk sac and embryo body at this differentiation stage.

Self Renewability and Immunoglobulin Heavy-Chain Expression of B-Cell Precursors Isolated from Embryos at Day 9 of Gestation. The time course of development of B cells from progenitors found in yolk sac and embryos from day 9 of gestation is shown in Fig. 1. It is readily apparent that cells from this stage of gestation require more time in culture to express their B-cell potential than do cells from latter-stage fetal liver. This observation raised the possibility that progenitors found at day 9 might undergo more rounds of cell division in an uncommitted state. The experiments summarized in Table 2 were designed to evaluate (*i*) the state of heavy-chain immunoglobulin commitment of the cells isolated from yolk sac and embryo bodies at day 9 of gestation, (*ii*) the period of time cells can undergo division without differentiation to immunoglobulin heavy-chain-committed pre-B cells, and (*iii*) the frequency of cells capable of expansion at a given time point.

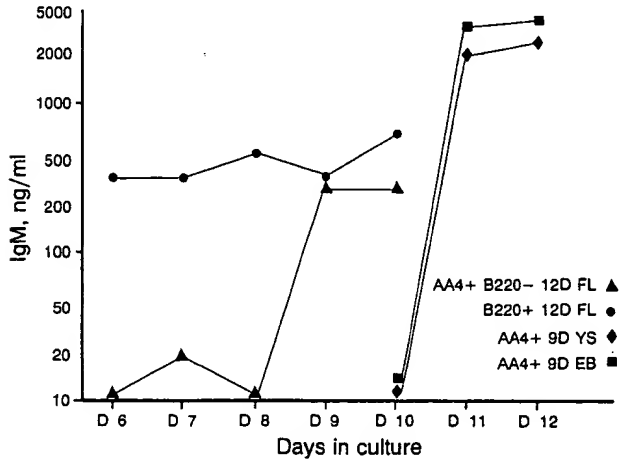

FIG. 1. Level of immunoglobulin secreted by the progeny of B220$^+$, B220$^-$ fetal liver (FL) cells, and day-9 AA4$^+$ embryo body (EB) and yolk sac (YS) cells. Cells were cultured at 10^4 cells per ml with IL-7 and S17 cells for the indicated number of days, after which they were transferred to a second plate (10^4 B220$^+$ cells in two wells, each containing 100 μl, of a 96-well plate) containing LPS and irradiated S17 cells. After 12 days in the secondary plate, the amount of IgM in 400 μl of supernatant was determined by ELISA. Results are expressed as ng of IgM per ml.

Table 2. Heavy-chain immunoglobulin commitment in B-cell precursors present in yolk sac and embryo bodies at day 9 of gestation

Day of cloning	Frequency of B-cell progenitors	No. of clones analyzed/no. LPS-responsive (ratio)	No. of clones expressing both Igh6a and Igh6b allotypes (ratio)
0	YS 1:30	14/14 (1)	14 (1)
0	EB 1:30	17/17 (1)	17 (1)
10	YS 1:1.8	23/19 (0.82)	13 (0.56)
10	EB 1:1.1	35/33 (0.94)	29 (0.82)
14	YS 1:3	30/19 (0.63)	10 (0.33)
14	EB 1:2.7	30/18 (0.6)	11 (0.36)

AA4.1$^+$ cells from yolk sac (YS) and embryo body (EB) of (C57BL/6 × BALB/c)F$_1$ mice were cloned on the day they were isolated (day 0) at 6, 20, and 60 cells per well in the presence of S17 stromal cells and IL-7. After 18 days in culture, the clones were stimulated with LPS, and the immunoglobulin secreted in the supernatant was analyzed for IgM of the Igh6a or Igh6b allotypes. The remaining cells were set in bulk culture on day 0 with the same stimuli at 5 × 10^4 cells per well in 2-ml cultures. On day 10 and 14 after initiation of culture a sample of cells was collected, and cells were cloned at 0.5 and 2 cells per well. After 8 days, clones were stimulated with LPS, and supernatants were analyzed as described above. Cells were stained at the time of recloning and shown >98% B220$^+$, as determined by FACScan analysis.

AA4.1$^+$ cells were isolated from yolk sac and embryo body (Table 2) of (C57BL/6 × BALB/c)F$_1$ mice. One aliquot was cloned under limiting-dilution conditions with S17 stromal cells and IL-7. The remaining cells were seeded in bulk cultures in the same culture conditions at 5 × 10^4 cells per 2 ml. Ten days after the initiation of culture the dominant cells in the cultures were small, nonadherent, and morphologically typical of lymphoid cells. Cell-surface analysis showed that >98% of these cells were B220$^+$ but failed to express IgM.

Clones started at day 0 of culture were all responsive to LPS 18 days later and secreted IgM of both Igh6a and Igh6b allotypes. This result indicates that virtually all B-cell precursors detected at day 9 of gestation are uncommitted for heavy-chain immunoglobulin expression (Table 2). By contrast, cloning of the LPS-responsive cells in the cultures at day 18 showed that these cells have become committed during the culture period to the expression of one or the other allotype (Table 3). Clones started on day 10 of culture were tested for LPS responsiveness 8 days later. Between 80 and 90% of these clones could respond to LPS, and 60–80% produced IgM of both heavy-chain allotypes (Table 2). Approximately 40% of the clones started on day 14 of culture still retained the ability to give rise to both IgM allotypes. We

Table 3. Cells that respond to LPS are restricted to the expression of one heavy-chain allotype

Frequency of LPS-responsive cells	Clones analyzed, no.	Clones expressing Igh6a and Igh6b allotypes, no.
YS 1:5	43	0
EB 1:4	40	0

After 18 days in culture, cells from the experiment described in Table 2 were cloned directly in LPS in the presence of S17 cells at 3, 12, and 36 cells per well. Supernatants were tested for IgM of the Igh6a and Igh6b allotypes. YS, yolk sac; EB, embryonic body.

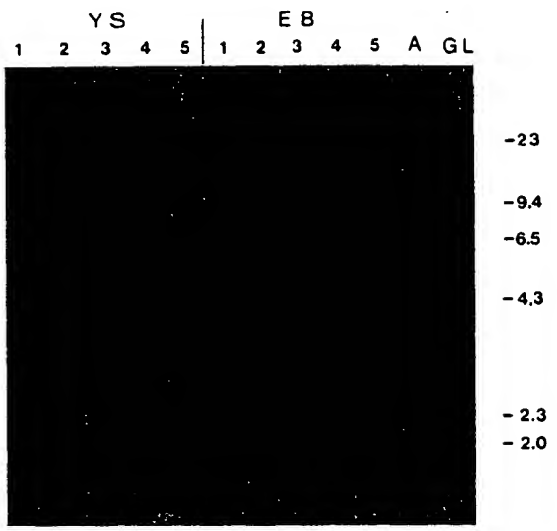

FIG. 2. Southern blot analysis of DNA isolated from independent clones expanded from AA4.1$^+$ precursor cells in the yolk sac (YS) and embryo body (EB) of embryos at the 9th day of gestation. Ten to twenty micrograms of genomic DNA was digested with the enzyme *Eco*RI, blotted onto nylon membranes, and hybridized to a JH4 probe labeled with ^{32}P by random priming. Lanes: A, DNA from an Abelson-transformed pre-B cell line; GL, liver DNA; 1–5, DNA from five clones from either the yolk sac or embryo body. Cells were >99% B220$^+$ at the time of DNA preparation.

also observed that fewer cells were LPS-responsive at this stage.

Individual clones of B-cell precursors isolated from yolk sac and embryo body could be expanded to clone sizes of up to 5×10^6 cells. Five clones from both tissue origins were expanded, and genomic DNA was isolated. After digestion with the enzyme *Eco*RI, Southern blots were probed with a JH3-4 probe. Those conditions detect all rearrangements in the immunoglobulin heavy-chain locus. As can be seen in Fig. 2, multiple bands can be detected (between 10 and 20) in each individual clone, confirming that extensive immunoglobulin gene rearrangements occurred during the period of culture.

Multiple Heavy-Chain Variable-Region (V_H) Genes Can Be Expressed by the Progeny of Single B-Cell Precursors. The pattern of immunoglobulin-variable-region expression is known to depend partly on the differentiation stage. In particular, it is well-documented that the initial rearrangement events use variable regions 3′ of S107 more frequently

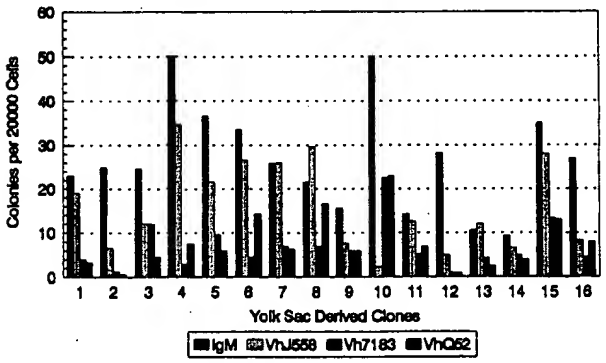

FIG. 3. V_H gene expression in B-cell colonies from 16 independent yolk sac clones. Bars show the number of IgM-, VhJ558-, Vh7183-, or VhQ52-expressing colonies per 2×10^4 cells plated in agar culture. Each bar is the mean of three replicate filters.

than predicted based on numbers alone (19). We analyzed the expression of three V_H gene families in the progeny of individual clones. The families analyzed were 7183 and Q52 gene families, the members of which lie 3′ of S107, and the J558 gene family, the members of which lie 5′ of S107 (20). Individual clones of progenitor cells isolated from day-9 embryos were expanded in the presence of S17 stromal cells and IL-7. Cells were then cloned in a double-layer agar culture, as described, stimulated with LPS for a period of 5 days (16). Fig. 3 shows the results of this analysis and contains representative clones from four individual experiments. Extensive use of each of the three families was observed. Nonetheless, clonal variation was apparent. For example, clone 10 contained very few J558-expressing cells, whereas clone 4 was dominated by the J558 family.

DISCUSSION

Fetal liver and fetal thymus are populated by hematopoietic precursors on the 10th–11th day of gestation. Before this time, hematopoietic precursors are found in the blood islands of the yolk sac. Myeloid progenitors that form colonies after exposure to appropriate growth factors have been routinely detected (10). Yolk sac also contains progenitors that can reconstitute both the myeloid and lymphoid compartments of irradiated mice, at least for several months (1, 3). However, it has been difficult to detect B-cell progenitors that can differentiate *in vitro* into immunoglobulin-secreting cells. This result was somewhat surprising because primitive progenitors found in other sites were able to undergo *in vitro* differentiation.

In this report we describe the isolation of B-cell precursors from the yolk sac as early as day 8.5 of gestation. Moreover, we show that B-cell precursors that can differentiate to immunoglobulin-secreting cells can be detected in the yolk sac and embryo at the same time. Ogawa *et al.* (9) reported that progenitors able to generate B cell in a similar *in vitro* culture assay arose first in late day-9 embryos. Such progenitors were not found in early day-9 embryos and only arose in yolk sacs on day 10. They concluded that the first progenitors arose in the embryo rather than in the yolk sac. Our results show that both the yolk sac and embryo harbor significant numbers of progenitors on day 9 of gestation. Small differences in assay conditions or in the mouse strains studied [BALB/c vs. (C57BL/6 × BALB/c)F_1] may account for these conflicting results.

We encountered considerable experimental variability in the number of progenitors found in the yolk sac or embryo. Further, there was no correlation in the numbers detected in single experiments between the yolk sac and the embryo. In some cases the numbers were much higher in one tissue and were reversed in other experiments. The variable results might reflect that we are detecting the initial emergence of cells able to respond in our assay. This notion is reinforced by the results obtained earlier than day 9, where we consistently detected B-cell progenitors but at frequencies less than one per yolk sac or embryo body. The results suggest that the progenitors either arise or expand at about the same time in both sites. This result does not exclude the possibility that a more primitive progenitor, not yet committed to hematopoietic differentiation, originates in a single site and subsequently seeds both the yolk sac and embryo.

We compared the progenitor cells found in the yolk sac and embryo based on surface markers, self-renewal potential, ability to differentiate *in vitro*, and the distribution of three immunoglobulin V_H families found in their mature B-cell progeny. In all these respects the yolk sac and embryo progenitors appeared identical. All B-cell precursors detected in this report possessed sufficient AA4 to be isolated by panning. By contrast, they do not express sufficient Ly6 to be depleted or enriched by panning. In this respect these

progenitors are distinct from those found in 12-day fetal livers that expressed both of these surface markers. There are several possible explanations for this observation. It might indicate that AA4 cell-surface marker appears earlier in hematopoietic differentiation than does Ly6. Alternatively, the different sites of detection may induce or favor the expression of different cell-surface markers on the same population of cells. Finally, the results may indicate that, for reasons that remain obscure, a different cell population, temporally confined to this short period of development, is being detected.

The precursors found at day 9 of gestation require more time in culture to mature to the LPS-responsive stage than do the precursors in the 12-day fetal liver. In fetal liver, at day 12 of gestation, most B-cell precursors are uncommitted for immunoglobulin heavy-chain expression. In addition, half of them are at least bipotent—able to give rise to both B cells and macrophages (13). Time-course experiments revealed that at least 8 to 9 days of *in vitro* growth and differentiation were required before the first LPS-responsive cells emerged from the fetal liver-derived precursors. Similar experiments with progenitors from yolk sac and embryo at 9 days of gestation revealed that an additional 48 hr was required for these cells to reach a similar point in differentiation. In our previous work, there was a strong correlation between the length of the required *in vitro* interval and the stage of differentiation. This result suggests that the progenitors derived from earlier embryos or yolk sac might, indeed, be more primitive. Experiments to determine whether there is a correlation between the time interval and the potential to give rise to multilineage progeny are now required.

One interesting feature of the B-cell precursors analyzed here is the capacity of extensive cell division without commitment to immunoglobulin allotype expression. After 10 days of *in vitro* expansion, 98% of the cells are $B220^+$, cell numbers have increased 50- to 100-fold, and still 60–80% of the cells that retain the ability to generate immunoglobulin-producing B cells are not yet allotype committed. During the ensuing 4 days (day 10–14) approximately half of those cells will become committed for heavy-chain allotypes.

The period of commitment to heavy-chain expression in these cultures (day 10–14) is also accompanied by a decrease in the frequency of cells that become LPS-responsive. This result indicates that there may be a selection for less-committed cells that continue to expand in response to IL-7 and S17 and may, therefore, dilute more mature precursors that have a reduced growth rate. This selection might also involve immature cells that have been arrested in their capacity to differentiate either by down-regulation of recombination activity or by the accumulation of nonproductive immunoglobulin gene rearrangements.

Because the B cells detected in this assay emerged from very primitive progenitors, it was of interest to determine whether they would display a restricted immunoglobulin heavy-chain V_H repertoire. We used the colony blot assay to test the expression of three common V_H families: J558, the members of which lie predominantly on the 5′ side of the immunoglobulin region, and two 3′ families, 7183 and Q52. We found that each clone tested contained progeny expressing each of the three V_H families tested. This result demonstrates that the precursors could differentiate and use multiple variable-region genes, a result consistent with our observation that these clones generally contain multiple immunoglobulin heavy-chain allotypes.

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